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Hetero-bivalent Imaging Agents for Simultaneous Targeting Prostate-Specific Membrane Antigen (PSMA) and Hepsin

PRINCIPAL INVESTIGATOR:
Youngjoo Byun, Ph. D.

CONTRACTING ORGANIZATION:
Korea University Research and BusinessFoundation
Yeongi-gun, Chungnam 339-700, South Korea

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Youngjoo Byun, Ph. D.

E-Mail: yjbyun1@korea.ac.kr

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14. ABSTRACT

The hypothesis of the original proposal was to improve the sensitivity and accuracy of prostate cancer diagnosis by targeting simultaneously PSMA and hepsin, which are highly expressed in advanced and metastatic prostate cancer. In Year 2, we successfully established the solid-phase synthetic strategy of PSMA-hepsin peptide conjugates and synthesized key intermediates to conjugate with imaging probes including optical dyes and radionuclides. The synthesized dual-targeting conjugates consist of PSMA-binding ligand (Lys-urea-Glu) and hepsin-binding ligands derived from the IPLLVVPLGGSSK peptide and indole-5-caboximidamide. In silico docking studies of compound library with human hepsin identified potential hits which can be utilized as hepsin-binding scaffolds. The synthesized conjugates will be labeled with radionuclides/optical dyes and evaluated in vivo in Year 3.

15. SUBJECT TERMS
PSMA, Hepsin, Prostate Cancer, Molecular Imaging

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A. Introduction

Prostate cancer (PCa) is the leading cancer in the U.S. population and the second leading cause of cancer death in men. (1) An estimated 241,740 new cases and 28,170 deaths of prostate cancer will occur in the US during 2012, according to the statistics of American Cancer Society (www.cancer.org). Even if current detection methods of PCa using prostate-specific antigen (PSA) testing have advanced significantly for the diagnosis of patients with PCa, the controversy on PSA is currently still being debated. There have been several recent clinical reports whether PSA testing is an efficient biomarker in diagnosing the prostate cancer and reducing prostate cancer deaths. Two studies in the European countries showed that men who receiving PSA screening can lower the risk of death from prostate cancer while the study in the USA did not show any difference between PSA screening group and non-PSA group statistically. (2-4) Due to the lack of PSA specificity for PCa, there has been an increase of unnecessary biopsies/treatments of what would be benign or indolent disease. Therefore, there is an urgent need to explore new biomarkers for early and precise detection of PCa, in particular small lesions, i.e., recurrent tumors in the surgical bed, local lymph node invasion and other subtle manifestations of the disease in men. PSMA (prostate-specific membrane antigen) and hepsin are on the line as clinical biomarkers of PCa due to the fact that they are highly expressed in metastatic prostate cancer

PSMA is a type II integral membrane metalloprotease that has abundant and restricted expression on the surface of prostate carcinomas, particularly in androgen-independent, advanced and metastatic disease. (5,6) PSMA possesses the criteria of an ideal target for immunotherapy and diagnosis, i.e., expression primarily restricted to the prostate, abundantly expressed as protein at all stages of the disease, presented at the cell surface but not shed into the circulation, and association with enzymatic or signaling activity.(6) SPECT-CT scan of PCa using ^{111}In-capromab pendetide (Cyt-356, ProstaScint), a [^{111}In]-labeled monoclonal antibody to PSMA, showed promise in the clinic for identifying candidates for salvage radiotherapy. (7, 8)

Because of the important functions of PSMA for PCa, we have designed and prepared PSMA-based imaging probes to evaluate its biological activity *in vivo* in a variety of conditions using PET, SPECT and optical dyes. (9) According to the recent PSMA crystal structures in complex with several ligands, the PSMA active site consists of two distinct sub-pockets, which form a 'glutamate-sensor' S1' site and an amphiphilic S1 site. The cylinder-like ~ 20Å deep tunnel region exists adjacent to the S1 site and penetrates the surface of the enzyme.(10) We have taken advantage of the structural freedom provided by the S1 pocket to introduce imaging moieties. The lysine in the S1 binding site was chosen as a core scaffold in order to 1) take advantage of the many radiohalogenation methods and radiohalogenated prosthetic groups developed previously for reaction with the ε-amino group of lysine residues, and 2) increase the structural diversity of urea-based PSMA inhibitors. (10, 11) Among several lysine-urea-glutamate (Lys-urea-Glu) analogs, 2-[3-[1-carboxy-5-(4-[^{125}I]iodo-benzoylamino)-pentyl]-ureido]-pentanedioic acid ([^{125}I]DCIBzL, IC_{50}=0.01 nM) and [^{99m}Tc]L1 (IC_{50}=10 nM) exhibited the promising properties for PSMA imaging *in vivo*.(10, 11) Armed with X-ray information and molecular modeling dynamics, we have also demonstrated that the bulky labeling groups including radiometal-chelators and optical-dyes can be conjugated with the Lys-urea-Glu moiety by linking with an optimal spacer, which occupies the > 20Å tunnel region. (10) The bulky SPECT/Optical PSMA-

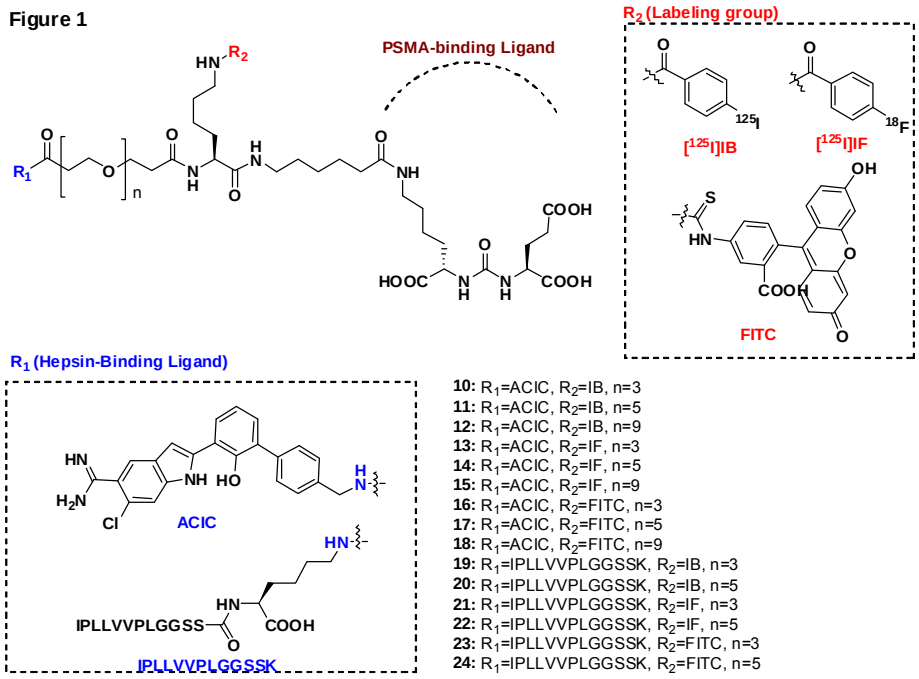
imaging probes with a spacer length of > 20Å demonstrated potent PSMA-inhibitory activities *in vitro* as well as selective uptake into PSMA-positive tumors *in vivo.* (12)

Hepsin also exhibited staining predominantly in the plasma membrane and was preferentially expressed in neoplastic prostate over benign prostate.(13) In addition, the mRNA level of *hepsin* was elevated in ~ 90% of PCa specimens and was > 10-fold higher in metastatic PCa than in normal prostate or benign prostatic hyperplasia (BPH). Hepsin is composed of 413 amino acids and a 373-residue is in extracellular region. The extracellular 255 amino acids at the C-terminus, so called serine protease domain, are highly homologous among typeII trypsin-like serine proteases.(14) There have no report on hepsin inhibitors with a nanomolar IC_{50} value, which is considered as a cut-off value of promising compounds for *in vivo* molecular imaging. Valency is the number of separate connections that one microscopic entity makes with another (16). In general, higher valency yields higher affinity. Recently, Kelly et. al reported the successful detection of prostate cancer at the *in vivo* animal studies using hepsin-targeted multivalent nanoparticles. (17) Moderately-potent peptide (IPLVVPL, 120 nM) conjugated with fluorescent-labeled nanoparticle improved binding affinity/avidity for hepsin and exhibited fluorescent signal *via* FACS by >10-fold higher than the peptide alone.

Our hypothesis of the original proposal was that more sensitive and potent PCa imaging agents can be developed by dual-targeting of PSMA and hepsin which are highly expressed in PCa, each with an extracellular active site. Their cell expression and high expression in the tumors, easy access of imaging probes to the target site and high affinity for PCa-cells through heterobivalency can be possible. The proposed imaging probes contain a PSMA-binding ligand and a hepsin-binding ligand linked by an optimal spacer, and will possess enhanced affinities/avidities for PCa compared to the corresponding monovalent PSMA or hepsin ligands.

B. Specific Aims in Year 2

Figure 1



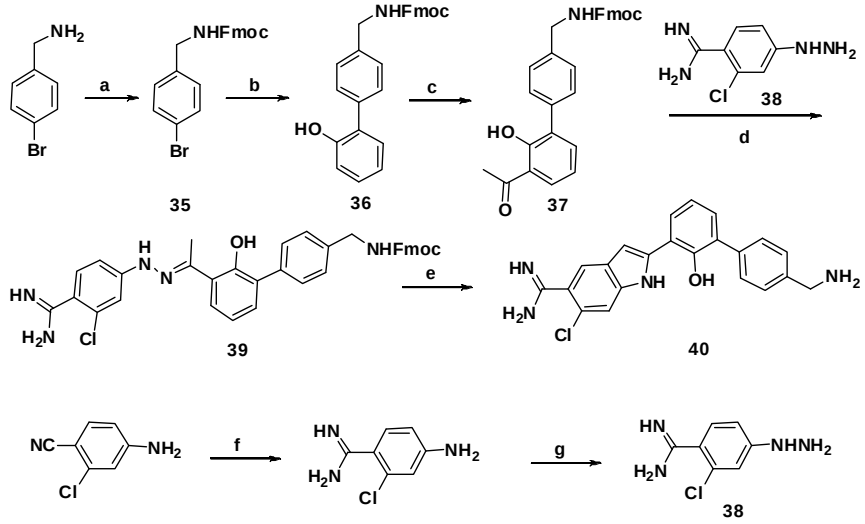
10: R_1=ACIC, R_2=IB, n=3
11: R_1=ACIC, R_2=IB, n=5
12: R_1=ACIC, R_2=IB, n=9
13: R_1=ACIC, R_2=IF, n=3
14: R_1=ACIC, R_2=IF, n=5
15: R_1=ACIC, R_2=IF, n=9
16: R_1=ACIC, R_2=FITC, n=3
17: R_1=ACIC, R_2=FITC, n=5
18: R_1=ACIC, R_2=FITC, n=9
19: R_1=IPLLVVPLGGSSK, R_2=IB, n=3
20: R_1=IPLLVVPLGGSSK, R_2=IB, n=5
21: R_1=IPLLVVPLGGSSK, R_2=IF, n=3
22: R_1=IPLLVVPLGGSSK, R_2=IF, n=5
23: R_1=IPLLVVPLGGSSK, R_2=FITC, n=3
24: R_1=IPLLVVPLGGSSK, R_2=FITC, n=5

We hypothized that the sensitivity and accuracy of PCa diagnosis can be improved by dual-targeting of PSMA and hepsin. We proposed heterobivalent conjugates of PSMA/hepsin-binding ligands labeled with optical dyes, positron- and gamma-emitting nuclides, in order to provide agents with enhanced affinity/avidity for PCa. The PSMA-binding ligand moiety of proposed conjugates is Lys-urea-

Glu, which has been used as the key S1'-probing moiety for preparation of our PSMA imaging probes. We proposed three hepsin-binding ligands based on the small molecules reported as weak to moderate hepsin inhibitors. These are indole-5-carboximidamide scaffold (ACIC) crystallized with hepsin (17) and IPLLVVPL peptide obtained by phage-display microarrays.(16) The ultimate goals throughout the 2-yr project period was to synthesize the heterobivalent conjugates of the PSMA-ligand (Lys-urea-Glu) with two hepsin ligands (indole-5-carboximidamide and IPLLVVPL peptide) and to evaluate their *in vitro* biological properties for *in vivo* animal imaging studies with optical- or radionuclide-labeled conjugates in Year 3. During the 2nd year period, we established the synthetic strategies of ACIC and IPLLVVL peptides and successfully synthesized key intermediates for the synthesis of hetero-bivalent conjugates as shown in Figure 1. The hetero-bivalent conjugates consist of three moieties that possess distinct roles for simultaneous targeting of PSMA and hepsin: (1) a high-affinity urea-based PSMA ligand, Lys-urea-Glu, (2) hepsin ligands derived from indole-5-carboximidamide or IPLLVVPL peptide, and (3) a nucleophilic functional group to be coupled with radionuclides, i.e., ^{125}I or ^{18}F, or optical dyes.

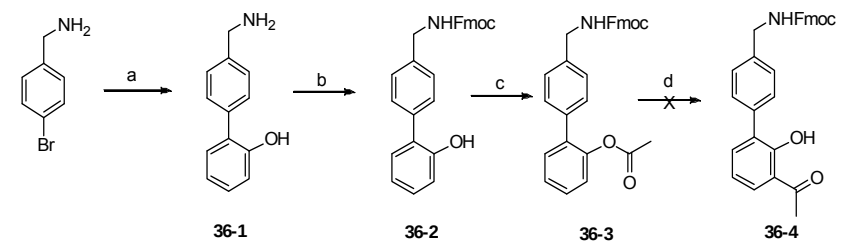
C. Accomplishments in Year 2

Scheme 1. Synthesis of ACIC



Reagents: (a) Fmoc-OSu, (b) 2-hydroxybenzeneboronic acid, $Pd(OH)_2$, (c) i: AcCl, pyridine/DCM, ii: $AlCl_3$, dichlorobenzene, (d) EtOH, TEA, (e) PPA, 155 °C, (f) NH4Cl, $Al(CH_3)_3$, p-xyelene, reflux, (g) $NaNO_2$, 6N-HCl

Scheme 2



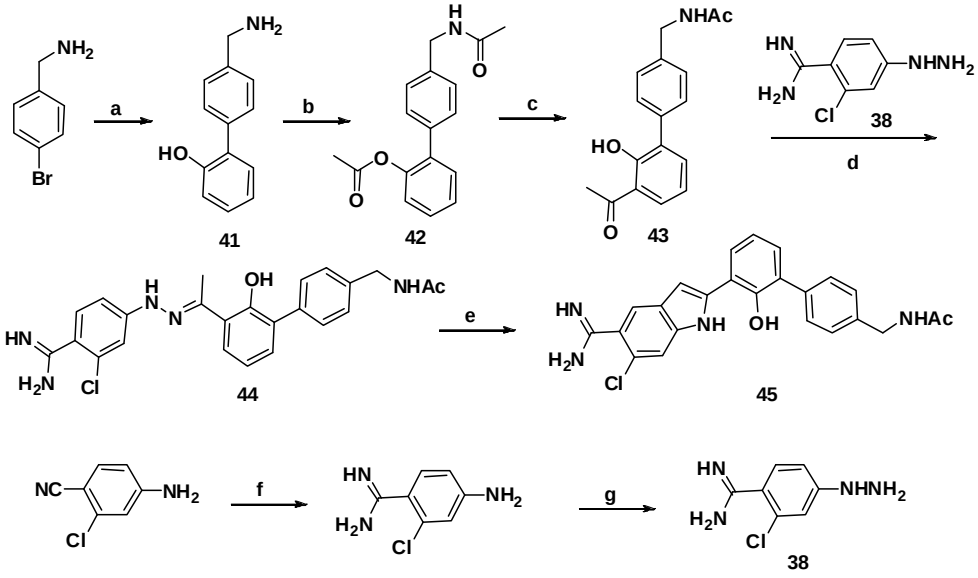
Reagents and conditions: (a) 2-hydroxybenzeneboronic acid, Cs_2CO_3, DMF/H_2O, Pd ($PPh_3)_4$, 100 °C, (b) Fmoc-OSu, DMF, (c) acetyl chloride, dichloromethane/pyridine (1:1), (d) $AlCl_3$, dichlorobenzene, 130 °C

1. Synthesis of ACIC-derived hepsin ligand

The original synthetic route to hepsin-binding ligand 2-(4'-(aminomethyl)-2-hydroxybiphenyl-3-yl)-6-chloro-1H-indole-5-carboximidamide (ACIC) is outlined in Scheme 1. ACIC is an analogue derived from a weak hepsin inhibitor (2-hydroxybiphenyl-3-yl-6-chloro-1H-indole-5-carboximidamide), of which crystal structure in complex with hepsin was reported. (18) Reaction of commercial 4-bromobenzylamine with Fmoc-OSu afforded the Fmoc-protected **35** in 69% yield. However, coupling of **35** with 2-hydroxybenzeneboronic acid *via* palladium-catalyzed Suzuki reaction gave **36** in low yield (< 20%) because of Fmoc-deprotection under the basic conditions. We investigated an alternative route to synthesize Fmoc-

protected 2'-hydroxybiphenyl analog **36-2** by employing the Suzuki coupling in the 1st step and subsequent Fmoc-deprotection as shown in Scheme 2. Compound **36-2** was prepared in a yield of 57% from 4-bromobenzylamine. Acetylation of **36-2** by the treatment of acetyl chloride afforded **36-3** in high yield. However, Fries rearrangement of **36-3** with $AlCl_3$ in dichlorobenzene at 130 ^oC did not afford the 2'-hydroxyacetophenone **36-4**. Ester moiety of **36-3** was hydrolyzed under Fries rearrangement conditions to regenerate **36-2**. We observed the same phenomenon in the 4'-hydroxybiphenyl analog, implying that bulkiness of Fmoc group may affect the rearrangement of the acetyl moiety. Therefore, we replaced Fmoc with acetyl ($COCH_3$) as protecting group of benzyl amine. The modified synthetic procedure of the hepsin-binding ligand 2-(4'-(aminomethyl)-2-hydroxybiphenyl-3-yl)-6-chloro-1H-indole-5-carboximidamide (ACIC) was outlined in Scheme 3.

Scheme 3. Modified synthetic procedure of ACIC



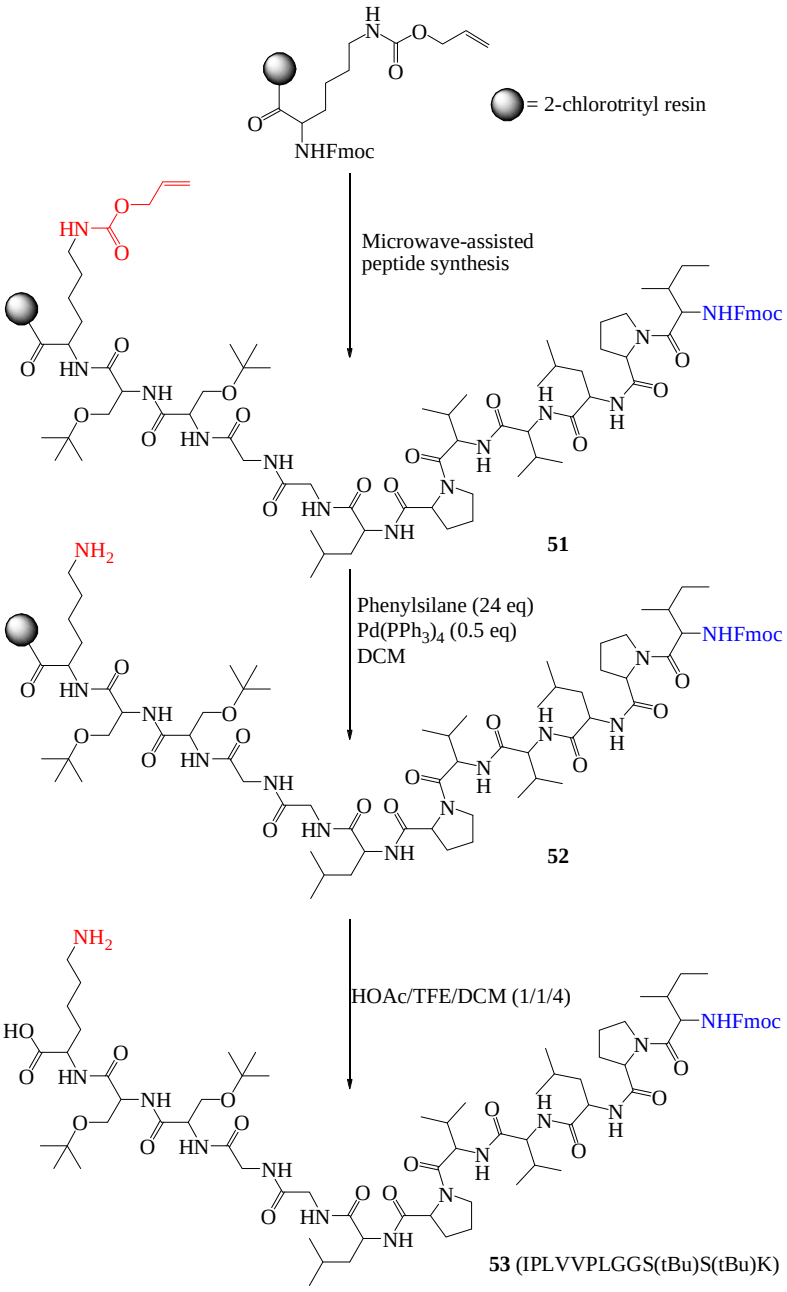
Reagents: (a) 2-hydroxybenzeneboronic acid, Cs_2CO_3, $Pd(PPh_3)_4$, DMF/H_2O, 100 ^oC, (b) AcCl, pyridine/DCM (1/1), (c) $AlCl_3$ (4 eq), dichlorobenzene, (d) EtOH, TEA, (e) PPA, 155 ^oC, (f) NH_4Cl, $Al(CH_3)_3$, p-xyelene, reflux, (g) $NaNO_2$, 6N-HCl

Suzuki-coupling reaction of 4-bromobenzylamine with 2-hydroxybenzeneboronic acid using $Pd(PPh_3)_4$ as a palladium-catalyst afforded the compound **41** in 78% yield. Acetylation of **41** by the treatment of acetyl chloride in pyridine/dichloromethane gave **42** in 62% yield. Fries rearrangement of acetyl moiety to the *ortho*-position occurred successfully by reacting **41** with $AlCl_3$ (2 eq) in dichlorobenzne at 130 ^oC for 24 hr. ^{1}H-NMR studies confirmed that the acetyl moiety of **43** appeared at 2.58 ppm while that of **42** at 2.11 ppm. 2-Chloro-5-hydrazinylbenzimidamide **38** was synthesized from 4-amino-2-chlorobenzonitrile in 2 steps in 35 % yield. Addition of 4-amino-2-chlorobenzonitrile to a mixture of ammonium chloride in p-xylene and trimethylaluminium (1M solution in n-heptane) at 0 ^oC, followed by reflux, resulted in formation of an amindine analog. The amidine was converted into the hydrazine **38** by the treatment of $NaNO_2$ and subsequent $SnCl_2$. Condensation of **43** with the hydrazine **38** gave asymmetric hydrazone **44**. Fisher indolization of **44** mediated by polyphosphoric acid (PPA) afforded **45** in 30% yield. After deprotection of acetyl group of **45,** coupling of **45** with **32** will give ACIC-based conjugates **10**-**18**, which are planned in Year 3.

2. **Synthesis of conjugate of PSMA with IPLLVVPLGGSSK-peptide ligand**

2.1. Hepsin-targeting IPLLVVPLGGSSK

Scheme 4. IPLLVVPLGGSSK synthesis

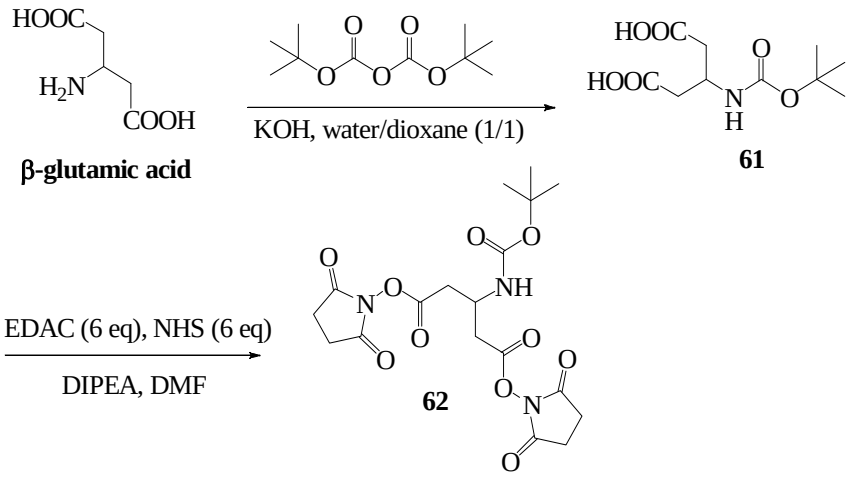


The IPLLVVPLGGSCK peptide, which has an affinity of 190 nM for hepsin-expressing PC3 cells (HPN-PC3 cells), was identified by phage selection method by Kelly et al. (17) The conjugate of the peptide with nanoparticles increased binding affinity for HPN-PC3 cells compared to the peptide alone. Compound IPLLVVPLGGS(^{t}Bu)S(^{t}Bu)K was prepared as shown in Scheme 4. Microwave-assisted peptide technology has been successfully applied for the synthesis of IPLLVVPLGGS(tBu)S(tBu)K. The allyloxycarbonyl (Alloc) protecting group of C-terminus lysine was used as a starting material. The amine group in the ε-position of the lysine was intended to conjugate with PSMA-binding ligand while that in the α-position of the lysine or N-terminus isoleucine with the imaging prosthetic groups such as optical dyes and radionuclides. The alloc group was removed by the treatment of phenylsilane and tetrakis(triphenylphosphine)palladium in dichloromethane. Treatment of **52** with a mixture of acetic acid/trifluoroethanol/dichloromethane (1/1/4) for 2 hr removed the key intermediate **53**, IPLLVVPLGGS(tBu)S(tBu)K which was confirmed by ESI-mass spectrum (M+H peak: 1501.8).

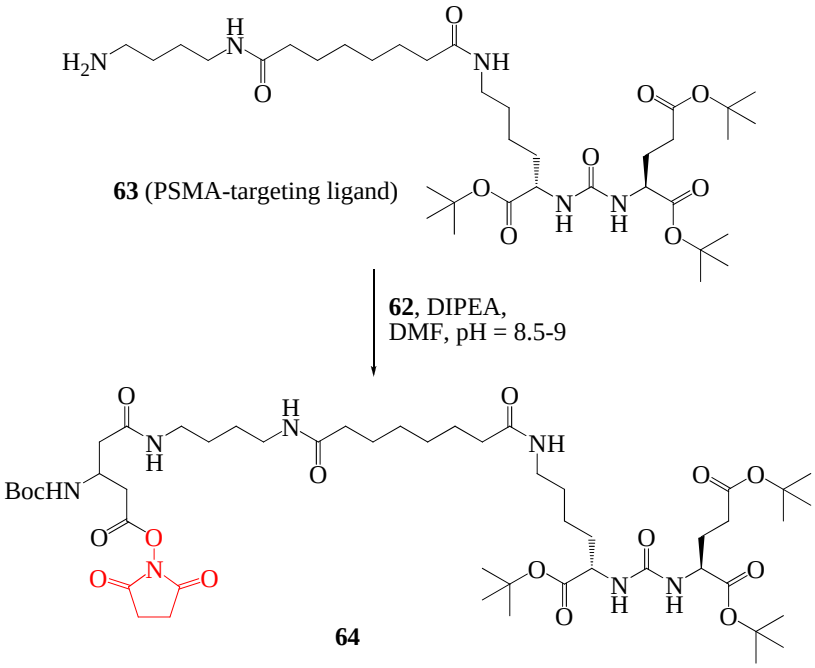
2.2. **Synthesis of Boc-β-glutamic acid-bis-NHS ester**

The β-glutamic acid shown in Scheme 5 was chosen as a linker of the PSMA/hepsin-targeting moieties as well as a functional group to couple with an imaging probe in order to avoid the formation of structural isomers. The two carboxylic acid groups of β-glutamic acid are chemically equivalent whereas those of α-glutamic acid are not. The amine group of β-glutamic acid was protected with Boc by reacting β-glutamic acid with Boc anhydride in water/dioxane in a yield of 63%. N-hydroxysuccinimide (NHS) activated ester was synthesized by reacting **61** with NHS using EDAC as a coupling agent in 60% yield. ^{1}H-NMR and ESI-MS confirmed the Boc-β-glutamic acid-bis NHS ester **62**.

Scheme 5. Boc-β–glutamic acid-bis-NHS ester



Scheme 6. The synthesis of PSMA-NHS ester

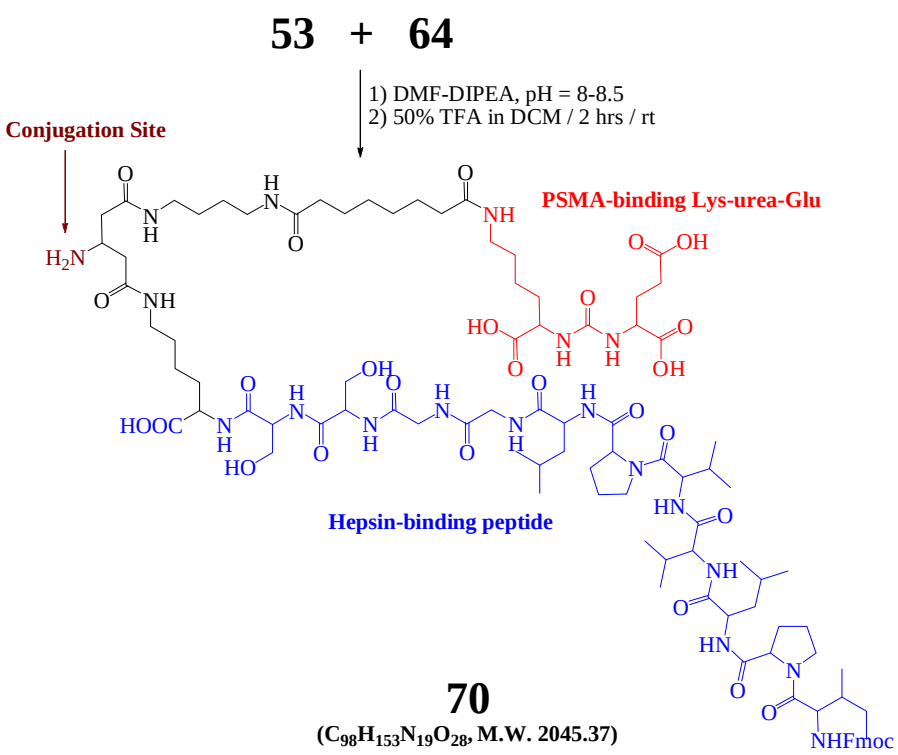


2.3. The synthesis of PSMA-NHS ester

The PSMA-NHS ester **64** shown in Scheme 6 is a versatile intermediate that can be used to couple with ligands of a variety of target proteins such hepsin and extracellular metalloproteases. Compound **63** was synthesized by following the procedure we have reported. (10, 12) Conversion of **63** into **64** was successfully achieved under basic conditions (pH: 8.5-9.0) in DMF. Reaction of PSMA-amine **63** with 3 equivalents of Boc-β-glutamic acid-bis-NHS ester **62** afforded **64** in 42% yield without any formation of dimer products. ESI-MS confirmed the formation of **64** with $[M+H]^{+}$ peak at 1041.2.

2.4. The synthesis of PSMA-Hepsin Conjugate (Scheme 7)

Scheme 7. Synthesis of PSMA-hepsin conjugate



Conjugation of **53** with **64** was achieved under the basic condition (DIPEA in DMF, pH=8.0-8.5). Subsequent removal of ^{t}Bu by the treatment of trifluoroacetic acid (TFA) afforded the target compound **70** which contains PSMA-binding moiety (Lys-urea-Glu) and hepsin-binding moiety (IPLLVVPLGGSSK). The free amine (brown color in Scheme 7) of **70** will be utilized for coupling with optical dyes or radionuclides in Year 3. ESI-MS spectral data confirmed the molecular $[M+H]^+$ ion peak at 2064.4 at the positive mode (see Figure 2).

Reaction of **70** with N-succinimidyl-4-[^{125}I]iodobenzoate(S[^{125}I]IB) or N-succinimidyl-4-[^{18}F]fluorobenzoate(S[^{18}F]FB) in the presence of TEA will provide the corresponding radiolabeled analogs for *in vivo* imaging studies in Year 3. For the synthesis of non-radiolabeled compounds, nonradioactive SIB and SFB will be used instead of S[^{125}I]IB and S[^{18}F]FB at the final step. In the similar way, optical dyes such as LI-COR dye (near infra red fluorescence dye) and FITC will be conjugated with **70** to produce optical imaging probes for *in vivo* studies.

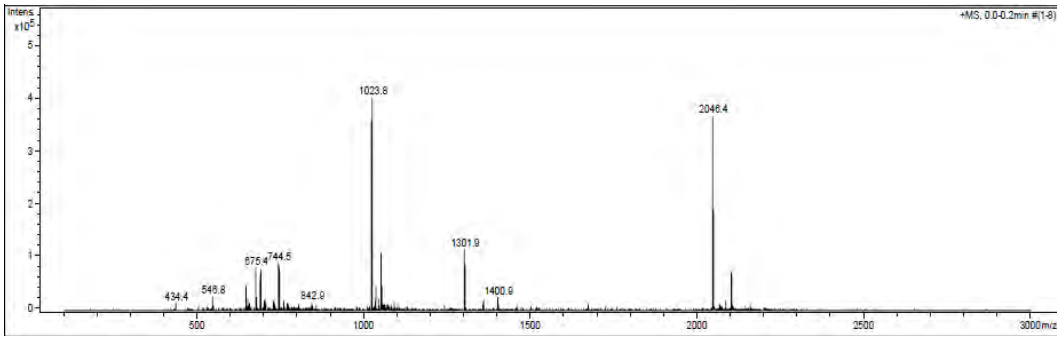
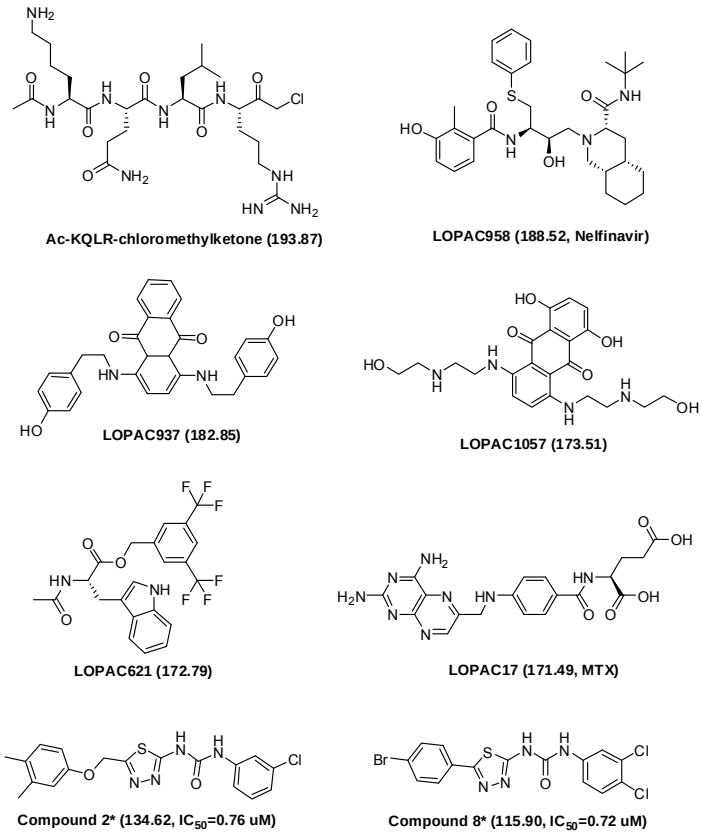


Figure 2. ESI-MS spectrum of compound **70**

3. *In silico* virtual screening of compound library with human hepsin

Figure 3. Representative his from in silico docking studies of compound library with hepsin



* IC_{50}s of compounds 2 and 8 were adapted from the data published by Chevillet et. al.

Compound library docking studies with human hepsin protein were performed by using Discovery Studio 3.0 program (DS 3.0, Accelrys Inc.). The crystal structure of human hepsin (PDB ID: 1Z8G) in complex with the peptide-derived ligand (Ac-Lys-Gln-Leu-Arg-methylketone) was utilized as a template. (19) The compound library consists of LOPAC^{1280} (pharmacologically active compound set from Sigma-Aldrich) and the published hepsin inhibitors. (20) The database file of 1,280 compounds of LOPAC^{1280} and 16 known hepsin inhibitors were generated in sd/sdf format. Ligand conformations were randomly generated and their energies were minimized using ligand preparation module. For docking studies, the hepsin protein structure was downloaded from the Protein Data Bank site. Water molecules and the original peptide ligand (Ac-Lys-Gln-Leu-Arg-methylketone) were removed for docking studies. All hydrogen atoms were added by application of CHARMm force field and Momany-Rone partial charge calculation. LibDock module of DS 3.0 is compatible for the high-throughput screening of compound library *in silico*. The default parameters (number of hotspots: 100, docking tolerance: 0.25) were used. Based on LibDock Score, the potential hit compounds were selected for *in vitro* screening and further structural modification of the identified hits will be planned in Year 3. More than 20 compounds from the LOPAC^{1280} showed the higher binding energies than the known hepsin inhibitors (compound **2** & **8** in Figure 3). Figure 3 summarizes the chemical structures of the representative hits with their *in silico* binding energies to hepsin. LOPAC 958 (Nelfinavir), a HIV-1 protease inhibitor, were as strong as Ac-KQLR-methylketone. Nelfinavir was reported to induce growth arrest and apoptosis of human prostate cancer cells such as LNCaP, DU 145 and PC-3 cells. (21) Anthraquinone-containing analogs (LOPAC937 and LOPAC1057 in Fig 3) exhibited high binding energies to

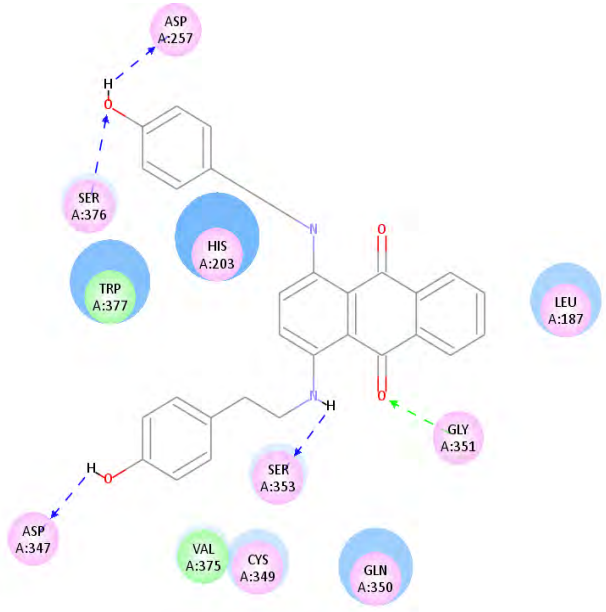


Figure 4. 2D-diagram of hepsin-LOPAC 937 interaction

hepsin, indicating that anthraquinone moiety can be utilized as scaffold to design new PSMA-hepsin conjugates. The 2D-diagram between LOPAC 937 and hepsin in Figure 4 showed that the LOPAC 937 interact the three amino acids (Ser 353, His 203 and Asp 257) in the triad system of the hepsin active site. Ser 353 and Asp 257 make strong intermolecular hydrogen bonds with amine and phenol moieties of LOPAC937, respectively. In addition, tryptophan-derived LOPAC621 and glutamate-derived LOPAC17 have higher binding energies compared to the reported hepsin inhibitors (compounds **2** & **8**), which can be utilized as hepsin-binding scaffolds in the future.

D. Research Plan for the 3rd Year

During the 2nd year of the award, we successfully established the solid-phase synthetic strategy of PSMA-hepsin peptide conjugates and synthesized key intermediates to conjugate with imaging probes including optical dyes and radionuclides. We plan to prepare labeled conjugates of PSMA-hepsin as imaging probes using the current key intermediate and to perform *in vivo* animal optical and SPECT-CT imaging studies in Year 3. In addition, we will synthesize new conjugates of PMSA with the hepsin ligands which identified from in silico docking studies. They exhibited strong binding affinities to hepsin compared to the low M.W. hepsin ligands which we designed in the original proposal. We believe that the continued funding for the 3rd year could allow us to get the point of viable, novel agents to image prostate cancer *via* dual-targeting of PSMA and hepsin. The principal investigator successfully established his own lab at Korea University (KU) and recruited the post-doctoral fellow and the graduate student who will carry out the synthesis, *in silico* docking studies, and *in vitro* assays. Animal imaging studies will be performed at Johns Hopkins University (JHU) under the direction of Dr. Martin G. Pomper. KU and JHU made the research agreement and the subcontract starting from 2012. With the continued funding in Year 3, we can accumulate the scientific data and publish them in a prestigious journal.

E. Milestone in Year 3: *Synthetic chemistry, radiochemistry and biological evaluation*

We will continue major on-going efforts to synthesize the non-radiolabeled conjugates of PSMA-hepsin at KU. Dr. Jianbo Chen and the graduate student at KU will carry out the synthesis, *in silico* docking studies, and *in vitro* assays under the supervision of PI. Dr. Chen joined Dr. Byun's lab in April, 2012 and got 100% salary support in Year 2 of this award. Because of his expertise and excellent performance, he was selected as a recipient of the salary-supported research professor program from KU and will receive 50% of his salary form KU from 09/01/2012 to 08/31/2013. Even if his salary-based FTE is 50% in Year 3, his dedication of 100% time efforts to the project will be continued. In Year 3, the remaining personnel budget will be allocated to the graduate student, who will join Dr. Byun's lab in November, 2012. During the Year 3 period (2012-2013), KU scientists will complete the synthesis of non-radiolabeled conjugates as well as their biological evaluations *in vitro.* JHU researchers will perform radio-synthesis and *in vivo* animal studies. Drs. Pomper and Mease at JHU, collaborators of this project, will carry out the radiolabeling of the PSMA-hepsin conjugates and *in vivo* molecular imaging studies.

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